oxo bile acids is beset with problems. Thus, oxo bile acids, in particular, 3-oxo bile acids, are vulnerable to rigorous alkaline hydrolysis conditions and therefore the much milder enzymatic hydrolysis of conjugates is preferred when oxo bile acids are suspected. Oxo bile acid methyl esters can be chromatographed without further derivatization (the hydroxyl groups in the partially oxidized compounds must be derivatized); however, sometimes artefacts are produced and it may be better to protect the oxo groups as the *O*-methyloxime or the dimethylhydrazone derivatives.

Summary

Detection and quantitation of bile acids are important in health and disease. Bile acid synthetic defects may arise from both defective cholesterol synthesis and metabolism; bile acid therapy may correct some of these abnormalities. GC has proved to be of vital importance in determination of bile acids in biological fluids. However, a complete GC analysis generally takes a long time. By appropriate column and derivatization selection, analysis time can be significantly reduced, but sample preparation for GC analysis is usually time-consuming. Combination of GC and mass spectrometry greatly aids in the characterization of unknown bile acids. In attempts to get additional information, Child *et al*. have developed a method to quantitate sterols and fatty acids together with bile acids in stool samples. Advantage is taken of the greatly increased retention times of the *n*-butyl ester-acetates of bile acids, so that sterol acetates are eluted earlier than all bile acids present and a group separation of faecal fatty acids, sterols and bile acids is achieved. In a further advancement of the method, Batta *et al*. have prepared *n*-butyl esters of bile acids directly in faecal samples, followed by trimethylsilyl ether formation. They showed that faecal bile acids could be quantitatively derivatized and eluted from the faeces and bile acids could be quantitated in the presence of other faecal components. The method is also applicable to plasma bile acid measurement. Figure 2 shows plasma and faecal bile acids in a healthy volunteer determined as the *n*-butyl ester-trimethylsilyl ether derivatives. Since the extra steps of extraction of bile acids and their purification are eliminated, the method can easily be adapted for routine screening of samples for bile acid analysis.

See also: **II/Chromatography: Gas:** Delectors: Mass Spectometry; Derivatization; **Solid Phase Extraction. III/Acids:** Gas Chromatography; Liquid Chromatography; Thin-layer (Planar) Chromatography. **Extraction:** Supercritical Fluid Extraction.

Further Reading

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Liquid Chromatography

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Introduction

Bile acids, the major components of the bile fluid, play a significant role in lipid metabolism. After formation in the liver and storage in the gall bladder, the bile is secreted in the duodenum. Due to their chemical structure, all bile acids tend to form micelles and serve as natural detergents. In addition, bile acids are co-factors of pancreatic lipases and are pivotal for the digestion and absorption of fats and fat-soluble vitamins.

The primary bile acids cholic acid and chenodeoxycholic acid are synthesized in the liver and during their enterohepatic circulation various modifications occur. Conjugation with the amino acids taurine or glycine as well as deconjugation, sulfation, glucuronidation and modification by intestinal

bacteria lead to numerous secondary bile acid structures. The biliary and serum bile acid pattern as such as well as the detection of several secondary bile acids in serum can serve as disease markers and are of interest in therapeutic monitoring.

In healthy individuals the bile acid concentrations in the gall bladder and in duodenal bile are in the upper micromolar up to millimolar range. The determination of faecal bile acids is, however, not hampered by their overall concentration but by the complex pattern of primary and secondary derivatives as well as by the nature of the matrix. Bile acid patterns in serum, which are for healthy individuals in the upper nanomolar concentration range, are much harder to determine.

A number of gastrointestinal disorders and hepatic or biliary diseases, e.g. liver cirrhosis and hepatitis, lead to increased serum and urinary bile acid levels and result in significant changes in the bile acid pattern. Since bile acids are the major products of cholesterol metabolism, they play an important role in research and development of lipid lowering drugs and therapies.

This is the reason why simple, reproducible and sensitive methods for detection, separation and quantitation of bile acid patterns in all kinds of biological matrices and body fluids are of increasing importance in the pharmaceutical industry as well as for clinical chemistry.

Liquid Chromatographic Methods

With enzyme-based assays or other analytical methods like radioimmunoassays either total bile acid concentrations or single bile acids can be detected. Gas–liquid chromatography is a commonly used method for bile acid analysis, however, it does not allow the detection of conjugated bile acids. Therefore, in order to achieve relevant diagnostic data with regard to changes in the bile acid pattern, other specific separation techniques have to be employed.

In early studies on liquid chromatography of bile acids, silica gel was a commonly used stationary phase. Other materials such as aluminium oxide or neutral polymers were also used for separations under gravity pressure. Various solvent systems, e.g. mixtures of light petroleum, butanol, glacial acetic acid and other organic solvents have been described, mostly in combination with thin-layer chromatography (TLC) as a detection method. With the systems described, the separation of different groups into conjugated/nonconjugated bile acids is possible, however poor sensitivity and insufficient selectivity are major drawbacks and make them unsuitable for routine diagnostics.

High Performance Liquid Chromatography (HPLC) Analysis

Separation

The development of sophisticated HPLC instrumentation and of precise pumps with low pulsation as well as improved sensitivity of the detection systems along with new column materials have made this technique well suited for routine laboratory work. Over the last 20 years, a number of different HPLC methods for determination of bile acids in all kinds of biological fluids have been published, each of them with special advantages and disadvantages.

Early HPLC work was based on polar column materials like silica gel and others. However, these were just adaptations from TLC and LC studies to higher pressure and were again limited in selectivity and reproducibility due to the nature of the matrix as well as the solvents used. It took the development of reversed-phase materials like octadecylsiloxane-modified silica to make HPLC the most commonly used analytical method in the bile acid field. Using nonpolar C_{18} materials, conjugated as well as nonconjugated bile acids can be separated satisfactorily. Reversed-phase materials tolerate most organic solvents and are stable over a pH range from 0 to 7. They also tolerate high pressures without changes in the column bed, and custom-made packing materials guarantee the required reproducibility of the analytical results. Therefore it comes as no surprise that almost all of the HPLC methods published for bile acid separation are based on reversed-phase applications.

The solvent systems described are normally based on organic solvents, e.g. acetonitrile or methanol, mixed with a buffer system. Buffers are based either on ammonium salts, ammonium acetate or ammonium carbamate, or on alkali phosphates.

Detection

The major challenge in bile acid analysis is their detection, particularly of unconjugated bile acids. Various methods have been described based on detection systems like UV absorption, refractive index, fluorescence, electrochemistry and enzymatic or chemical post-column derivatization. The most common and sensitive methods will be discussed here. Electrochemical and refractive index detection, due to their limitations in sensitivity and reproducibility, play only a marginal role in bile acid analysis.

UV detection The amino acids glycine and taurine make the whole group of conjugated bile acids detectable by UV absorption at 200 nm (**Figure 1**). This method limits the components of the mobile phase to

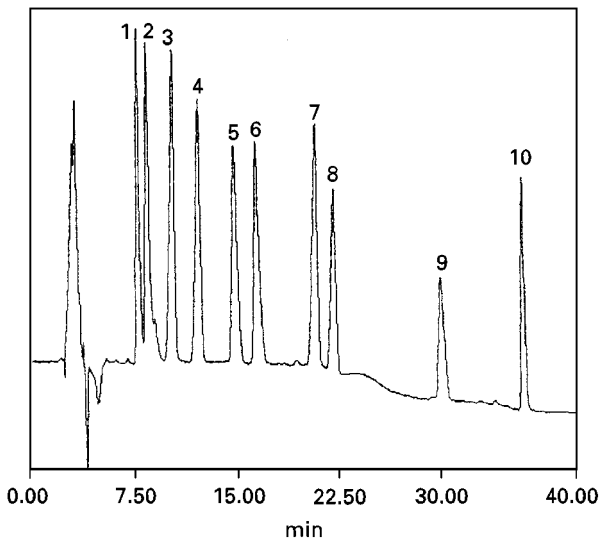

Figure 1 Separation of 10 conjugated bile acids by reversed-phase HPLC and UV detection at 200 nm: 1, taurocholic acid; 2, tauroursodeoxycholic acid; 3, glycocholic acid, 4, glycoursodeoxycholic acid; 5, taurochenodeoxycholic acid; 6, taurodeoxycholic acid; 7, glycochenodeoxycholic acid; 8, glycodeoxycholic acid; 9, taurolithocholic acid; 10, glycolithocholic acid.

solvents with low UV absorption. As detection at low wavelength is sensitive to all kinds of impurities, efficient sample preparation especially of biological material with low bile acid concentration is recommended. Also high purity reagents for the preparation of buffers and ultra pure organic solvents are required. Taking this into consideration, all methods based on RP-HPLC with UV detection allow qualitative and quantitative separation of all conjugated bile acids with biological relevance by gradient elution. The detection limits are in the range from 40 to 60 ng and show some individual characteristics for the respective bile acids.

Lacking a characteristic chromophor and due to their chemical structure, unconjugated bile acids cannot be determined by UV detection with sufficient sensitivity under conditions comparable to their conjugated counterparts. One solution to this problem might be the addition of an ionic compound like hyamine®, tetrabutylammonium phosphate or decyltrimethylammonium bromide as counter ion to the mobile phase. Separation and detection are then based on the formation of ion pairs. Some reports suggest that this method increases the sensitivity as well as the selectivity of separation and detection so that UV determination of unconjugated bile acids in a range of 20–30 ng is possible. In contrast to that, detection limits for the conjugated forms are about three-fold lower. However, since other detection methods provide higher sensitivity and efficiency, ion pair chromatography is rarely used in bile acid analysis.

Fluorescence detection A method to increase sensitivity in determination of bile acids is fluorescence labelling and fluorescence detection. Two different systems to generate fluorescent bile acid derivatives are commonly used.

A relatively old method to quantify total bile acid concentrations is enzymatic detection by 3α-HSD. Since all natural bile acids possess a hydroxyl group in the 3α-position, by enzymatic dehydrogenation and via formation of nicotinamide adenine dinucleotide (NADH), spectrometric detection at 340 nm is possible. Going back to this principle, 'column reactors' containing immobilized 3α-HSD have been developed. When connected with the separation unit, usually a C_{18} column, each substance is enzymatically transformed and as one of the reaction products, NADH can be determined by fluorescence detection at 350–460 nm. This method allows detection of nearly all free bile acids having a 3α-hydroxyl group independent from conjugation, but is not suitable for derivatives where the 3α-hydroxyl group is esterified, e.g. by sulfation or glucuronization. In the analysis of biological samples, other steroids with free 3α-hydroxyl group can influence the results. Application of the post-column derivatization method needs additional instrumentation for introduction of the enzymatic reagent.

On the other hand, several fluorescent agents for pre-column derivatization have been reported. One of the most reproducible and sensitive methods is based on 4-bromomethyl-6,7-dimethoxycoumarin (BMC) as derivatizing agent BMC reacts with carboxyl groups by formation of fluorescent esters detectable at 340–460 nm. Therefore, unconjugated and glycine-conjugated bile acids can be labelled as well as all kinds of bile acid esters at the 3α-hydroxyl group. Despite the high sensitivity – concentrations of less than 5 ng can be detected – the method has disadvantages. One major problem results from the fact that taurine-conjugated bile acids cannot be detected, due to the lack of a carboxyl group necessary for an ester bond. Samples containing high amounts of these conjugates, e.g. gallbladder bile, have to be treated with choloylglycylhydrolase for deconjugation before the total amount of the free acids can be measured, or an additional method for detection of taurine conjugates has to be employed. Though the BMC reagent will react with all components possessing a free carboxyl group, e.g. fatty acids, bile acids can be well separated and quantified by choosing an appropriate elution system and adequate sample pretreatment. The BMC derivatization reaction works with dicyclohexanocrown ether (DCCE). Because of that, potassium salts of the bile acids have to be prepared so that a further sample preparation step

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can be carried out. An alternative, very sensitive method with a detection limit of less than 1 ng has been suggested, based on the formation of naphthyl ester derivatives. This labelling procedure also needs some careful preparation steps in order to obtain a good analytical sample for fluorescent detection. Nevertheless, pre-column fluorescence labelling seems to be suitable especially for samples containing low total bile acid concentrations, but also for samples with unconjugated bile acids as major components as in serum (**Figure 2**).

Detection by mass spectrometry The most sensitive technique by far for bile acid detection is the coupling of a suitable HPLC system with a mass spectrometer. Detection limits range from 1 to 5 pg. However, the prerequisite for the production of valid and reproducible data is high quality equipment, resulting in high initial costs. LC-MS coupling is the method of choice for the specific determination of low bile acid concentrations in all biological matrices or tissues. Mass spectrometry requires very small sample volumes and flow rates. For the HPLC separation a microsystem able to produce constant flow rates in the range of microlitres per minute is recommended. The use of standard HPLC systems is possible, but a precise flow-splitting device has to be used. This has the advantage that the main part of the eluate can be utilized for further determinations, e.g. UV detection, in order to achieve additional data for quantification. A high performance system allowing the baseline separation of all bile acids of interest is necessary to obtain best results from MS detection. Both isocratic and gradient elution are possible, and all kinds of bile acids, despite conjugation or other modification, can be detected. After sample pretreatment and purification to remove interfering substances no further steps such as deconjugation, derivatization or enzymatic treatment are required.

Different mass spectrometry systems are reported to be suitable for LC-MS coupling. HPLC-thermospray MS methods were reported earlier, but seem to be less sensitive and reproducible compared to other methods. Fast atomic bombardment (FAB) MS and electrospray ionization (ESI) MS have been interfaced with HPLC and good results in reproducibility and sensitivity have been reported.

A special type of a mass detection system is the evaporative light-scattering mass detector (ELSD). A special feature of this detection method is that the eluate coming from the column is evaporated, and nonvolatile components such as bile acids are determined by measurement of the light deflected. The detection unit consists of a photodiode and a laser beam. When nonvolatile compounds pass through the laser, light scattering appears and can be detected. The signal is dependent on the molecular mass of the separated substance. The ELSD technique seems to be moderately sensitive with respect to UV detection but detects all bile acids and their derivatives as well as other analytes of interest. However, isocratic elution systems are required and, therefore, ELSD detection is only applicable in niche areas of application.

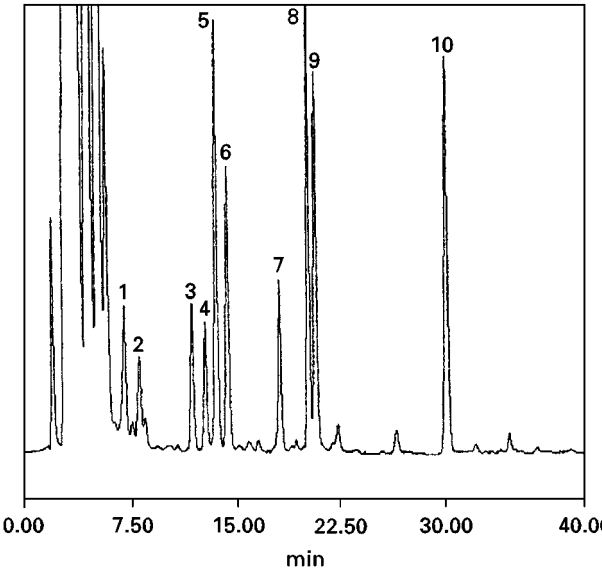

Figure 2 Separation of 10 unconjugated and glycine conjugated bile acid derivatives (pre-column fluorescence derivatization with bromomethoxycoumarin) by reversed-phase HPLC and fluorescence detection: 1, glycoursodeoxycholic acid; 2, glycocholic acid; 3, glycochenodeoxycholic acid; 4, glycodeoxycholic acid; 5, ursodeoxycholic acid; 6, cholic acid; 7, glycolithocholic acid; 8, chenodeoxycholic acid; 9, deoxycholic acid; 10, lithocholic acid.

Detection of Bile Acids in Biological Samples

Bile The determination of the bile acid pattern in gallbladder or intestinal bile does not need a time-consuming sample pretreatment or enrichment. The bile fluid of mammalian individuals consists mainly of glycine- and taurine-conjugated bile acids in relatively high concentrations compared to other body fluids such as serum or urine samples. Therefore, HPLC separation with UV detection is the commonly used method for analysis of samples derived from bile. In the bile fluid of healthy individuals only small amounts of protein are present and do not interfere with bile acid detection. Therefore, no deproteination procedure is necessary. However, in order to remove small amounts of protein as well as cell debris and larger particles, the addition of methanol followed by a centrifugation step is recommended. For conventional analysis, dilution with the mobile phase and filtration through a microfilter is sufficient as sample pretreatment. The use of a guard column in the HPLC

system is recommended when larger sample quantities are to be analysed.

Faeces Problems in the analysis of bile acids in faeces are not caused by the total amount of bile acids but by the complexity of the faecal matrix. Since the primary bile acids in gallbladder bile and in the intestinum are modified by the intestinal microflora during enterohepatic circulation, in faeces mainly secondary unconjugated bile acids are found. Time-consuming extraction procedures have been suggested, e.g. Soxhlet extraction or the use of alcoholic alkali solutions. Homogenization of the material in a mixture of chloroform and methanol using an ultraturrax mixer, followed by further purification with solid-phase extraction cartridges for sample pretreatment as described for serum preparation, seems to be able to extract most faecal bile acids quantitatively. Methods for pre-column fluorescence labelling, e.g. with bromomethoxycoumarin derivatives, are reported. For these derivatization procedures, sample pretreatment with choloylglycylhydrolase for deconjugation is recommended. In addition, detection with an evaporative light-scattering detector can be applied, with the advantage that a mass sensitive detection system is not limited by modifications in bile acid structure. However, the method suffers from nonspecific matrix products influencing the signal to noise ratio and hampering bile acid detection.

Serum and urinary samples The extraction, detection and quantification of bile acids in serum samples of healthy individuals is the most ambitious goal in the field of bile acid analysis. However, since changes in serum pattern can be characteristic for several metabolic and cancer diseases, considerable efforts have been made to develop methods of high sensitivity and reproducibility as tools for diagnostic purposes. Due to the nature of the matrix and to total bile acid concentrations in the low micromolar range, a sophisticated sample pretreatment procedure is absolutely necessary. The commonly used method for extraction and purification of serum samples employs solid-phase extraction as described by Setchell *et al*. Reversed-phase C_{18} cartridges are activated with methanol and water as recommended by all manufacturers of these columns. Sodium hydroxide solution is added to the serum samples and is followed by a 15–30 min heating step where samples are incubated at 64°C to suppress protein binding. The resulting solution is passed through the activated C_{18} cartridge where the bile acids should be retained while the matrix components are found in the eluted solution. Washing steps with water, in some cases followed by nonpolar organic solvents such as n-hexane, are carried out. Bile acids are recovered by elution with methanol. In most cases, eluted samples are dried and redissolved in a small volume of the mobile phase used for HPLC separation. In some reports an additional purification step by anion exchange cartridges is described.

Despite the fact that this sample preparation method has been commonly used over a number of years it suffers from several disadvantages. The major problem is the relatively strong binding of bile acids to serum proteins. Incubation at higher temperatures leads to protein denaturation but does not release the bile acid binding sufficiently and reproducibly. Another approach is the enzymatic digestion of serum proteins. However, this method seems to be limited, since the products of the protein digestion, small peptides and amino acids, interfere with further HPLC detection. Recent work describes a deproteination procedure by adding serum samples slowly to acetonitrile and incubating them in an ultrasonic bath for some minutes. This efficiently prevents co-precipitation of proteins and bile acids and secures reliable and validated analytical data. Another point has to be considered if solid phase extraction is applied as a sample pretreatment. C_{18} column materials are offered by several manufacturers and differ in their quality and their respective features. For instance, endcapped and non-endcapped C_{18} phases are offered commercially. These materials can differ in the quantity and the selectivity of bile acid retention. This leads to remarkable differences in bile acid pattern for the sample when different C_{18} cartridges are used. Therefore, the results obtained can only be validated when exactly the same material, i.e. same specifications, batch, etc., is used.

An interesting method for automation of the sample pretreatment procedure has been described by Yoshida *et al*. The online purification, concentration and separation of bile acids in serum samples is performed by an HPLC device equipped with three columns. First samples are injected on to a hydroxyapatite column to remove serum proteins. For further purification and concentration, a second pre-column (Serumout-25®) is used. After washing and elution from this column the bile acids are injected via an injection valve to the reversed-phase separation column. This strategy of sample pretreatment might be of value for high throughput assays, e.g. in clinical diagnostics. However, it leaves the problem of protein binding unsolved and, therefore, lack of sensitivity and efficiency is expected.

In urinary samples almost the same problems as in serum bile acid analysis occur. In contrast to blood samples, the urine from healthy individuals does not contain proteins and is available in much

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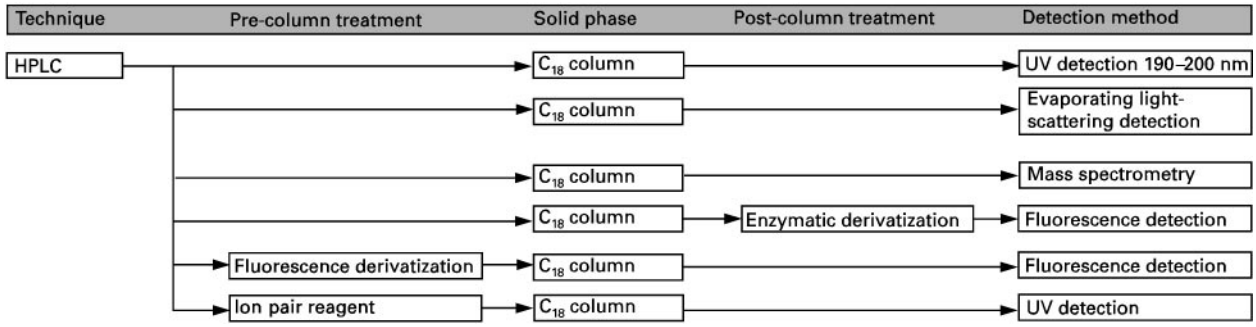

Figure 3 Separation methods for bile acids by liquid chromatography.

higher quantities. For these samples the standard purification procedure using C_{18} solid-phase extraction is suitable.

Due to the low concentration range of the bile acid pattern in serum and urine, the methods of choice for quantitative and reproducible determination are pre-column fluorescence derivatization and HPLC-MS coupling.

Conclusion

With regard to the liquid chromatographic methods described for bile acid analysis, especially with regard to their determination in biological matrices, several approaches for qualitative and quantitative determination have been described. A critical evaluation of the advantages and disadvantages of these methods results in the conclusion that there are specific limitations for every application (**Figure 3**). Since cumbersome derivatization steps as well as laborious and unsuitable sample pretreatment play a key role in the value of the analytical results, technologies are needed where direct analysis of the bile acid pattern, especially in serum, is feasible. Only HPLC-MS coupling presently allows such analysis, but costly equipment and highly skilled personnel requirements limit this method to highly specific problems and makes it unsuitable for routine work.

Presently, HPLC of bile acids is state-of-the-art and only incremental improvements can be expected from new column materials and microcolumns ($\varnothing$ 1–2 mm). The future will be in the area of low cost, high throughput HPLC-MS devices for routine use.

See also: **II/Chromatography:** Liquid: Derivatization. Detectors: Evaporative Light Scattering; Detectors: Fluorescence Detection; Detectors: Mass Spectrometry; Detectors: Ultraviolet and Visible Detection. **III/Bile Acids:** Gas Chromatography.

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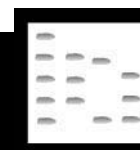
BILE COMPOUNDS: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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Introduction

Gall bladder bile is, like other physiological fluids, of very complex nature. It is produced by liver